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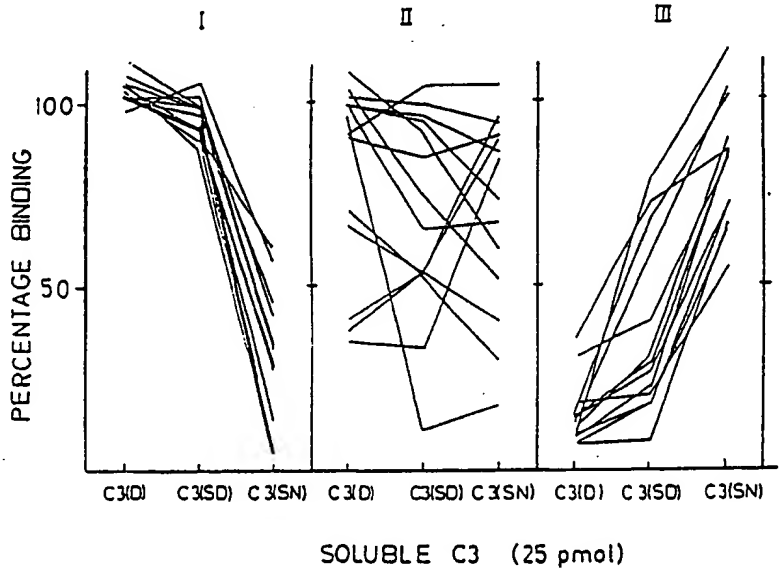

(57) Abstract

Antibody preparation directed against an individual neoantigen in the C3b region of human C3, and the use and production thereof. The preparation is characterized by immunnochemically reacting with (a) antigenic determinants in the C3b region of the alpha or beta chain of denatured human C3 in which the disulfide bonds have been reduced, but not with (b) human native C3. The use comprises immunochemically binding the preparation to C3 fragments, especially C3 fragments bound to immune complexes. The production encompasses the raising of an immune response containing the antibodies and selecting from said response the antibodies having the specificity given above.

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Antibody preparation directed against neoantigens in human C3 (complement factor 3) and the use and manufacture thereof

The invention is concerned with monospecific antibody preparations directed against so-called neoantigens (= neodeterminants) in human C3 (= complement factor 3). The preparations are in the first place of the monoclonal type. Their use involves immunochemically binding anti-C3-fragment-antibody-active components thereof to the appropriate C3 fragment; this is done especially for the purpose of detecting C3 fragments, in particular C3 fragments bound to circulating immune complexes (= CIC).

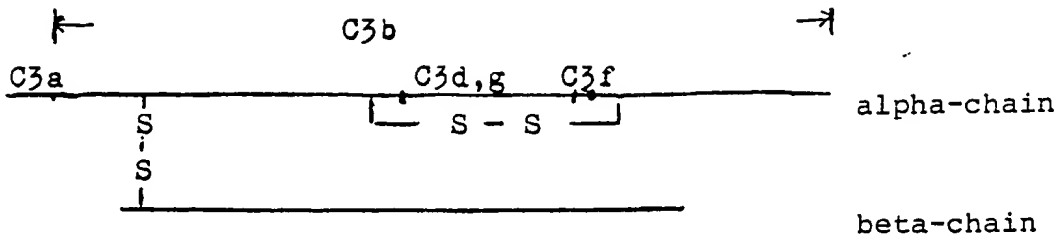
The term "C3" as used hereinafter refers to human C3 unless otherwise stated. Immunoglobulin is designated "Ig".

The complement system consists of about 20 components which have to react in a well-defined reaction sequence in order that destruction of e.g. invading microorganisms can take place in the final stage. The complement system is considered to be a fundamental element of the mammal's defense against infections caused by bacteria and by viruses. The components discovered first were called "factors" and were designated chronologically C1, C2 ..... C9 all along as they were being discovered.

Complement activation can often be correlated to inflammatory processes, exposure of the factors to synthetic surfaces, surgery and diseases involving immune complex formation, e.g. infections, autoimmune diseases, cancer etc.

C3 is composed of two different polypeptide chains. They are called the alpha chain and the beta chain, respectively, and are linked together by disulfide bonds. The alpha chain is believed to have a molecular weight of 115,000 daltons and the beta chain is believed to have a molecular weight of 70,000 daltons.

The structure of C3 is at present believed to be as follows:



There are several more S-S bridges, both inter-chain and intra-chain. Fragmentation sequences C3b —> C3bi + C3f; C3bi —> C3c + C3d,g.

It may be noted especially that the C3d region contains a thioester grouping.

C3 is believed to have a very complicated three-dimensional structure. Activation of C3 produces in a first step C3a and C3b, whereby the thioester group becomes accessible for reaction with hydroxyl or amino groups. C3b can thus be bound covalently to appropriate acceptors (target surfaces, substrates) having such groups, as e.g. cell walls, immune complexes etc. A competitive reaction is hydrolysis. C3b bound to an acceptor is capable of participating in the subsequent part of the activation sequence. C3b is inactivated by being fragmentized into C3bi (= iC3b) and C3f. C3bi may then in turn yield C3c and C3d,g. Fragmentation and/or binding to target surfaces will involve conformational changes so that new antigenic determinants are exposed with concomitant disappearance of original determinants. Antigenic determinants which are not to be found in native C3 but which do come forth in fragments thereof are called "neoantigens" ("neodeterminants"). This subject matter is dealt with in numerous review articles (5).

Major efforts have been made over the years to detect neoantigens and to make antibody preparations directed against them. Particular interest has been attached in this

context to neoantigens the generation of which is dependent on the binding of C3 fragments to various target surfaces, as for instance immune complexes. Antibody preparations directed against immunocomplexed C3 fragments have been considered to be useful for detecting C3-containing CIC and tissue-deposited C3 fragments (4, 31). Various methods for immune complex determinations have been compared (14, 15).

A number of monoclonals directed against C3 have been described. Tamerius, JD et al. (25, 26) have prepared monoclonals directed against human C3 (immunization with C3). Only one of these reacted exclusively with C3 fragments (C3d region). Lachmann, PJ et al. (10, 11, 12, 32) have described three different monoclonals. Only one of them (clone 9) reacted with neoantigen (in the C3g region). Burger, R et al (2) have produced eight different monoclonals by immunization with guinea pig C3. All the eight monoclonals reacted with native guinea pig C3 bound to plastic surfaces of microtiter wells, and two of them reacted moreover also with human C3 (clones 105 and 111). Aguado, MT et al. (1) have selected some of the aforesaid monoclonals for analyzing them in respect of their use for the detection of immune complexes and complement activation. Whitehead, AS et al. (29) have produced a monoclonal that reacts with native C3. None of the known monoclonals have been convincingly shown to discriminate between acceptor-bound fragment and the corresponding free form of the fragment.

Hansen, O. et al. (7) have described an adsorbed antiserum possessing a specificity directed against C3c epitopes. The preparation contains antibodies directed against a plurality of determinants.

The inventors themselves have shown in a number of earlier papers that determinants brought forth when C3b binds covalently to erythrocytes cell membranes can be found also in denatured C3 (16, 18, 19, 20). On the basis of these

findings the inventors have classified antigenic determinants of C3 as being (a) C3(S) which are stable determinants present both in native C3 and in denatured C3, (b) C3(D), these being determinants generated upon denaturation, and (c) C3(N), these being determinants present in native C3 and being upon denaturation replaced by C3(D). Examples of denaturing media apt to generate C3(D) determinants are SDS (= sodium dodecyl sulfate = sodium lauryl sulfate), deoxycholate, guanidine hydrochloride and acidic or alkaline media (pH <3 or pH >10, respectively). Polyclonal rabbit antibody preparations have been described which are directed against each respective type of determinant, i.e. C3(D) alpha, C3(D) beta, C3(D), C3(N), C3(S) and C3(S,N). The terms "alpha" and "beta" stand for each respective peptide chain of C3, that is reduced forms have been employed in the immunization procedure.

In an international-type search report compiled by the Swedish Patent Office in connection with the prosecution of the Swedish priority application, the references 1, 11, 16, 26, 32, 33 and 34 have been categorized to be of particular relevance, either alone or combined with other references.

Objects of the invention are to provide antibody preparations having an improved specificity against neoantigens (as compared to preparations previously known), in particular against neoantigens exclusive for bound forms which are generated due to covalent binding of C3b to a target surface or due to further fragmentation of C3b. Among other objects may be mentioned improved methods for detecting (a) soluble C3 fragments, (b) complement activation, (c) tissue deposition of C3 fragments, (d) C3 fragments bound to cells, (e) immune complexes containing C3 fragments etc. A third object is to provide alternative methods for diagnosticating the aforesaid conditions where complement is activated. A further object that may be mentioned, finally, is an improved method of obtaining antibody preparations directed against individual neoantigens.

The antibody preparations of the invention are monospecific against the individual neoantigens that are present in reduced forms of C3, in the first place in the C3b region of C3, such as the C3c or C3d region. The neoantigens in question are present either (preferably) in the alpha chain thereof or in the beta chain. An antibody preparation of the invention will thus not react with native intact C3 homologous to the immunogen used for immunization, but will react with denatured and completely reduced forms thereof, that is, with at least one of the C3 polypeptide chains dissociated from the other chain. In certain cases the preparation may also react specifically with at least one fragment (possibly bound to a target surface) selected from the group consisting of C3b, C3bi, C3c, C3d,g, C3d, C3g and other physiologically occurring C3b fragments. The preparation preferentially reacts with the fragments covalently bound to any one of the aforesaid target surfaces, but may in certain other cases also react with other forms. Particularly good are preparations with which it is possible to immunochemically assay for bound fragment in the presence of the corresponding free fragment.

The preferred antibody preparations of the invention are not significantly inhibited in their reaction with C3(D) by soluble (free) native C3b, C3bi, C3d,g or C3d fragments. For instance under the conditions given in the experimental part a more than 25 times higher, such as 50 times higher dose of the soluble fragments compared to the corresponding SDS-denatured or covalently bound fragments (molar basis) is required to effect the identical inhibition in the inhibition-ELISA.

The antibodies of the preparations may be present therein in the form of antibody-active fragments, for instance Fab, Fab', $F(ab')_2$. They may also be in the form of derivatized antibodies. The essential requirement is that the antibody fragments and the derivatives possess biospecific immune-type affinity in accordance with the present invention.

The antibody-active components of the preparations may be provided with analytically detectable groups such as enzymatically active, fluorogenic, chemiluminogenic, radioactive, biotinyl groups etc., or groups capable of acting as cofactors, coenzymes, substrates or cosubstrates etc. The components may be in a form where they are bound to a phase that is insoluble in the test medium - so-called solid phase; thus for example they may be physically or covalently bound to various polymers such as hydrophilic OH- or NH_2-containing polymers or plastics surfaces like those of microtiter wells. The preparations may be in the form of a solution or suspension to which have been added various known per se chemicals as required for any special type of use. The preparations may be freeze-dried; they may be packaged in tightly sealed packs for being employed as components of a test kit etc.

One aspect of the invention is a test-pack contemplated for the immunochemical assay of at least one type of C3 fragment. The test pack comprises an antibody preparation according to the invention.

For producing an antibody preparation according to the invention, cells potentially capable of producing antibodies of the specificity as prescribed in conformity with the invention are caused to secrete such antibodies which are then isolated, purified and optionally fragmentized and/or derivatized in a manner known per se. The purification involves removal of those antibodies that fail to fulfill the prescribed specifications. In a vertebrate, preferably a warm-blooded vertebrate (e.g. mammal such as mouse) secretion may take place in vivo as a result of immunization with an immunogen having the particular neoantigenic structures (neodeterminants) as contemplated here. The resultant immune response is polyclonal, so an antiserum obtained from the animal will contain antibodies directed against all the determinants of the immunogen, irrespective of whether or not these are neoantigenic. By employing suitable selection

methods the specificity of the immune response can be limited. By means of a suitable immunosorbent technique it is potentially possible to obtain purified forms of the antibodies directed against the desired neoantigen. In the present stage immunosorbent purification of polyclonal antibodies to obtain the preparation of the invention is a laborious procedure which will always give low yields.

The best method for selecting the antibodies in the immune response in order to obtain a good antibody preparation according to the invention is a so-called monoclonal technique (8) by which after immunization antibody-producing plasma cells are fused with cells of a suitable myeloma cell line so that they become capable of quick and uninterrupted growth. By cloning, selecting and culturing the fused cells that produce antibodies having the specificity (C3(D)) and cross reactivity in accordance with this invention it is possible to obtain antibody preparations directed against the individual antigenic determinants which are to be found in reduced forms of C3, i.e. in the free peptide chains thereof. Cultivation of the selected cell clones for producing the antibody preparations of the invention may be carried out in cell cultures in vitro or as ascites tumors in vivo. Purification and isolation may be performed in the same manner as purification and isolation of any antibodies in general - by salt precipitation or by means of various chromatographic methods like, for instance, ion exchange, affinity, gel etc. chromatography.

Immunogens that may be employed are those that will exhibit the aforesaid particular determinants when exposed in vivo to the immune system. A surprisingly large proportion of antibodies of the specificity as prescribed according to the invention are obtained in the immune response if the immunogen is either the alpha chain or the beta chain in the denatured form, a mixture of them, or suitable immunogenic fragments

of the chains. Examples of such fragments are denatured-reduced forms corresponding to the C3b, C3bi, C3d,g and C3c regions of these chains.

The antibody preparations of the present invention are useful primarily in immunochemical assay methodology for C3 fragments, but they may also be used potentially both in vitro and in vivo for modulating complement activation.

The assays comtemplated in the present context involve contacting a sample containing C3 fragment with the antibody preparations of this invention to thus form an immune complex the formation and amount of which are a quantitative measure and qualitative measure, respectively, of this C3 fragment in the sample. If required also additional reactants may be used which will react with components present in the immune complex formed (anti C3 fragment - C3 fragment). Practical considerations will decide whether these latter reactants are to be added before, after or simultaneously with the antibody preparation of the invention. Usually at least one of the reactants used is provided with a suitable marker group, a so-called "analytically detectable" group. Proportions of the reactants are then chosen such that the amount of labeled reactant incorporated in the complex or the amount thereof remaining unincorporated is a measure of the aforesaid C3 fragment in the sample.

According to one classification system, the methods may be classed as being either homogeneous or heterogeneous methods. Homogeneous methods assay for a labeled reactant without involving any physical separation of the labeled reactant incorporated in the complex from the non-incorporated form. When heterogeneous methods are employed the two forms of the labeled reactant are separated physically from each other before the labeled reactant is assayed for in either or both of the two forms. For ease of separation it is helpful if one of the reactants is insoluble in the test medium.

According to a second classification system, the methods may be classed as being either competitive or non-competitive methods. In a competitive method the arrangement is such that two immune reactants having a common epitope (determinant) are made to compete for an insufficient number of homologous binding sites on an immunological counterpart. In order to thus assay for a C3 fragment present in the sample and having a neoantigen against which a preparation of the invention is directed, the neoantigen of the sample is allowed to compete for the antibodies with an added reactant which carries the same neoantigen. The added neoantigen may be in a labeled, solid-phase-bound or soluble form.

Which particular form is to be chosen will be a matter subject to practical considerations, for example, with regard to the form in which the fragment is present in the sample. Competitive methods are often called "inhibition methods". In a non-competitive method arrangements are such that no competition can take place.

According to a third classification system, the methods may be classed as being either precipitation or non-precipitation methods. As examples of useful precipitating reagents may be mentioned precipitating antiserum and so-called solid-phase-bound antibodies, both of these directed against components of the complex, preferably against the antibody components. It may be mentioned in this context that neodeterminants in C3 as a rule are nonrepetitive; for this reason monoclonal antibodies directed against them are non-precipitating:

According to a fourth classification system, the methods are classed according to the marker group employed; the methods are thus radio-, enzyme-, fluorescence-, chemiluminescence-, enzyme-substrate-, immunochemical methods etc.

Among immunochemical methods may be mentioned also immuno-electrophoresis, particle agglutination, immunodiffusion and miscroscopy with labeled antibodies.

The exact choice of method is decided by the form in which the fragment sought is present in the sample. If the fragment is dissolved in the sample and is not bound to a target surface a method may be chosen in accordance with the principles applying to soluble analytes in general. If on the other hand the particular fragment sought is bound to a certain special target surface then this fact has to be taken into account when the method to be employed is chosen. Thus, the simplest way of assaying for tissue-deposited fragments is with the aid of an antibody preparation in a labeled form, optionally in combination with microscopy. Fragments bound to blood cells may be assayed for in an analogous manner, although for quantification purposes practical advantages are gained if recourse is had to inhibition techniques.

As a rule at least two reactants are required for selective assays for specific C3 fragments which are bound to specific soluble target surfaces (=·M). One of these reactants has to be of a type reacting specifically with a neoantigen in the fragment while the other reactant should react with a suitable epitope on the target surface. This implies methodologically that a ternary complex is formed (reactant 1 ---C3-M--- reactant 2). In the technical language this is called a "sandwich", and the general principles applying to "sandwich" tests can be applied when the specific method is chosen.

The invention is applicable in particular to a method of assaying for circulating immune complexes (CIC) having C3 fragments bound to them covalently. The complex thus contains (1) C3 fragment (C3b, C3bi, C3d,g, and C3d are those that are known up to now to exist as covalently bound forms), (2) antibody which may be of the IgA, IgD, IgE, IgG or IgM type, and (3) antigen. Known techniques are generally applicable, with the exception that an antibody preparation is used which has the specificity as prescribed according to the invention. Procedures will differ according to what exactly

is to be measured in each individual case. If the total amount of immune complex containing a certain fragment is to be determined then the general precipitation methods for immune complexes may be employed in combination with an antibody preparation of the invention which is specific against the particular neodeterminant present in the fragment sought. If an antibody preparation is employed which reacts with all conceivable C3 fragments potentially present in immune complexes, then the result obtained will be the total content of C3 fragments bound in the immune complex. If specific components of C3 fragment-containing CIC are to be assayed for, two reagents are required differing inter se by being specific for different components of CIC. The reagent required in that case in addition to an anti-C3-fragment-antibody preparation is a reagent that is specific for the antibody portion or antigen portion in the CIC. For example, the reagent may be specific for one of the known Ig classes or for the antigen. Optionally in addition to these reagents still further reagents may be used. Assays for immune complexes containing C3 fragments have been described heretofore, (1, 21, 23, 31).

The invention is applicable to assays in various types of samples which contain C3 and/or fragments thereof. It has been known that C3 and its fragments are present in, for example, tissues and body fluids like blood, plasma, serum, urine, synovial fluid, cerebrospinal fluid etc. C3-forms having no C3(D)-determinant can in certain cases be assayed, if they are denatured before reaction with the anti-C3-preparation.

Conditions employed for carrying out the immunochemical reaction(s) are such as are usual in this type of assay methods. For instance, temperatures may be chosen within the range of 0 - 40 ^{o}C, especially 15 - 40 ^{o}C. A suitable pH is usually pH 4.5 - 10, preferably about 5 - 8.6. Quite generally, of course, measures and steps have to be taken so as to avoid activation of complement or undesired denaturation of

C3. Complement activation requires the presence of proteases and divalent calcium and/or magnesium ions. It is therefore suitable to add protease inhibitors or agents that will form complexes with the said ions, like EDTA, if C3-forms carrying C3(D)-antigens are to be assayed in the presence of other forms. Detergents and buffer systems if added should then be of a kind that will not have a denaturing effect on C3.

The invention is further defined in the attached claims and will now be illustrated by means of examples which form a part of this specification and which are not to be construed as limiting the scope of the invention in any way.

MATERIALS AND METHODS

C3 preparations

Native C3 (C3(SN)) was purified as described previously (17). Denaturation of 100 µg of C3 (C3(SD)) was performed in 100 µl of 2 x 10^{-3}M SDS for 30 min at 37 °C. Denatured-reduced C3 and isolated C3 alpha and C3 beta chains (C3(D)) were prepared as in Nilsson et al. (19). Elastase-generated C3c and C3d were a kind gift from Dr Brian Tack, Scripps Clinic and Research Foundation, La Jolla, USA (24). C3b was prepared in the presence of 1 % (w/v) trypsin (TPCK treated, Worthington, USA) for 2 min at room temperature (17). C3bi was obtained by incubating C3b with 5 µg of factor H and 1 µg of factor I for 60 min at 37 °C. Radiolabelling of native C3 with ^{125}I was performed as described previously by a lactoperoxidase technique to a specific activity of 30 000 cpm/µg protein (16).

Antibody preparations

Polyclonal anti-C3-alpha-beta chain (anti-C3(D)) antibodies were raised in rabbits as previously described (19).

Mouse monoclonal antibodies

Group I: Mouse monoclonal antibodies raised against native C3 and C3b and selected for their specificity for soluble and erythrocyte-bound C3b were a kind gift from Dr Hans Müller-Eberhard, Scripps Clinic and Research Foundation, La Jolla, USA (25).

Group II: These antibodies were raised against and selected for specificity for SDS-denatured C3. Two Balb/c female mice, 8 - 12 weeks of age, were primed with 24 /ug of human SDS-denatured C3 in Freund's complete adjuvant (FCA, Behringwerke AG, W. Germany) together with 20 /ug of Lipopolysaccharide W (LPS, Difco, cat no 3120-25) subcutaneously. Eight weeks later, on day four before fusion, 100 /ug of SDS-denatured C3 in phosphate buffered saline, pH 7,4, was given intraperitoneally (i.p.). Hybridomas were produced according to standard procedures (6,9) with the following minor modifications. Four different Sp2/0 lines were used. Original Sp2/0.Ag14 (22) growing in standard Dulbecco's Modified Eagle's Medium (DMEM, Paisley, Scotland, cat no 041-1966) containing 5 % fetal calf serum (FCS, Gibco, cat no 011-6290) and 25 /ug/ml of Gentamycin (cat no G-7507, Sigma Chem Co, USA) was the progenitor line. Two different subclones selected for growth in serum-free medium and one subclone growing in low serum medium Hy-0.1 were also used. Standard DMEM containing 10 % FCS or HY-0.1 media were used for selection and cloning. The clones were screened for binding to SDS-denatured C3 in the direct-binding ELISA. Fourteen clones were randomly selected and further tested.

Group III: These antibodes were raised against and selected for specificity for denatured-reduced C3. Monoclonal antibodies in this group were produced in the same way but with the following modifications. Mice were injected s.c. with 30 /ug of denatured-reduced C3 in FCA. 30 /ug booster doses of the same antigen which was present in Freund's incomplete adjuvant (FIA, Behringwerke AG, W. Germany) were administered

i.p. four times at two week intervals, after which the animals were left alone for nine months. Final boosters with 190 /ug of denatured-reduced C3 in PBS were given i.p. once a day three days before fusion. A subclone of Sp2/0.Ag 14 myeloma line selected for growth in low serum medium Hy-0.1 were used in the fusions. Two different hybridoma lines were selected and cloned either in DMEM-5 % FCS or low serum medium Hy-0.1 %. The clones were selected for specificity in the direct-binding ELISA. 140 clones were obtained out of which 13 were randomly selected and further tested.

Established hybridoma lines were expanded by fed-batch type culture in TC flasks to 200 ml vol. The monoclonal antibodies were purified utilizing cation exchange chromatography as described earlier (3).

Clone selection for group III

An initial selection of monoclonals was effected by way of analyzing the reactivity to polystyrene-adsorbed denatured-reduced C3. 644 positive clones were detected.

In the second step of the selection process all the positive clones were compared with respect to their reactivities vis-à-vis seven different fragments and conformational variants of C3 in the direct-binding ELISA. What was found was a great variation in the reactivity patterns of the monoclonals. Representatives of each pattern were selected. Clones showing weak reactivities were excluded. In this manner 140 clones were selected, all of them strongly reactive against denatured forms of C3 but with varying specificity and reactive intensity against different types of C3 fragments. Of these then 70 clones could be expanded to a 200 ml culturing volume, and from this group then 14 were randomly selected for further specificity analysis. The ELISA tests were repeated and the Ig concentration was quantified.

One of the fourteen clones showed loss of reactivity and had stopped producing Ig. The others still had the same specificity patterns as before, with a strong specificity for C3(D) in solution which means they were directed against the neoantigens as per aforesaid definition. The presence of these neoantigens was then assayed for in physiological forms of bound C3. In order to avoid undesirable conformational changes and reduced access to the antigen surface the reactivity of the monoclonals was tested by means of inhibition ELISA and sandwich ELISA where the monoclonal antibody had been tethered to a solid phase by direct adsorption or indirectly via an anti-mouse Ig.

Preparation of particle-bound C3

$EAC14^{oxy}23b$ and $EAC14^{oxy}23bi$ cells were prepared and the uptake of C3 on the cells was estimated with ^{125}I-labelled native C3 as described before (16). 100 mg of boiled Zymosan A (Sigma Chem. Co., USA) was incubated with 2.5 ml serum containing 2 µg of native ^{125}I-labelled native C3 for 30 min at 37 °C. The uptake of C3bi was calculated from the particle-associated ^{125}I cpm.

Enzyme-linked immuno sorbent assay (ELISA)

Direct-binding assay: Serially diluted anti-C3 antibodies were allowed to bind to constant amounts of C3 or C3 fragments adsorbed to microtitre plate wells. The bound antibodies were thereafter quantified by anti-rabbit or anti-mouse immunoglobulins conjugated with horseradish peroxidase (HRP) (DAKO Immunoglobulins A/S, Denmark). Phosphate buffered saline (PBS) containing 0.1 % TWEEN 20 (v/v) and 0.1 % (w/v) bovine serum albumine (BSA) was used as a working solution.

1. 200 µl each of C3, C3 alpha and beta chains, SDS-denatured C3, C3c and C3d in PBS (corresponding to

20 nmol of C3/l) was adsorbed separately to the plastic surface of different wells of microtitre plates (Immuno-plate II F, Nunc, Denmark) overnight at 4 °C.

2. 100 µl of serially diluted antibody preparations was incubated in the wells for 60 min at room temperature (RT).

3. 100 µl of swine anti-immunoglobulins conjugated with HRP was allowed to bind to surface-associated anti-C3 antibodies from step 2 for 60 min at RT.

4. The enzyme reaction was started by the addition of 100 µl of colour reagent (20 mg of 1,2-phenylenediamine-dihydrochloride (Fluka AG, Switzerland) and 10 µl 30 % H_2O_2 in 75 ml of 0.1 m citrate/phosphate buffer pH 5.0) to the wells after step 3. The reaction was stopped by 100 µl of 1 M H_2SO_4 after approximately 10 min. The stain was quantified spectrophotometrically at 492 nm.

Following steps 1 - 3, the wells were rinsed extensively 3 times in saline containing 0.1 % TWEEN 20.

Inhibition assay: This assay was a modification of the direct-binding assay. 100 µl of serially diluted sample was allowed to compete with the adsorbed antigen for binding to a constant amount of antibody. The dose of antibody was selected to give OD_{492}=1 when binding to preadsorbed native C3 for 60 min at RT as described in the direct-binding assay. Following these initial steps the inhibition assay was completed by performing steps 3 - 4 of the direct-binding assay.

Sandwich ELISA

1. Indirect binding of monoclonal to microtiter wells.

An affinity purified Fc-specific rabbit anti-mouse Ig was adsorbed to microtiter wells (NUNC Immunoplate type I) in that 1.25 µg Ig/ml in 96 x 200 µl were incubated at +4 °C for about 16 hours or more. Then after washing the wells were incubated with a serial dilution of monoclonal (which may be unpurified supernatant), 100 µl/well, 1 hour at RT.

2. Immune complex analysis

After step 1 100 µl of a standard dilution from 1/10 to 1/10240 or plasma samples diluted 1/26 were incubated in the wells for one hour at RT. Next followed an incubation with an affinity purified Fc-specific rabbit anti-human IgG which was beta-galactosidase-conjugated and diluted 1/200 (1 hour at RT). The amount of enzyme bound to the wells was quantified with the aid of PRIST/RAST developing substance (100 µl, Pharmacia AB) which was allowed to react in the wells for two hours, whereupon the reaction was stopped after two hours with 50 µl of 0.66 M Na_2CO_3.

The wells were extensively rinsed 3 times after the incubations with the different antibodies and samples.

Immune complex model

Aggregated IgG with bound C3 was prepared in that serum is activated with an addition of 600 µg of aggregated IgG/ml and was incubated at 37 °C (14).

Samples

EDTA-plasma stored at -70 °C.

SDS-PAGE/Immunoblotting

SDS-PAGE was performed according to the method of Laemmli et al (13) and immunoblotting as described earlier (16). In

the immunoblotting technique anti-mouse immunoglobulin conjugated with HRP was employed to detect the monoclonal antibodies.

RESULTS

Specificity of polyclonal antibodies raised against denatured-reduced C3

The specificity of polyclonal rabbit anti-C3(D) antibodies and antisera raised in mice against SDS-denatured C3 (derived from production of group II) and denatured-reduced C3 (derived from production of group III) was characterized by ELISA. Serially diluted antibody preparations were tested in the direct binding-assay and all three types of antibodies were shown to bind to wells coated with native C3. Native C3 (S and N antigens), SDS-denatured C3 (S and D antigens) and denatured-reduced C3 (D antigens) were allowed to compete for binding to the three types of antibodies in fluid-phase in the inhibition assay (Fig. 1). As expected, rabbit anti-C3(D) antibodies were inhibited by C3(SD) and C3(D) but not by C3(SN) antigens confirming that C3(D) antigens were involved in their binding. In contrast, both types of mouse antisera were inhibited both by C3(SD) and C3(SN) but poorly by C3(D), the only difference being that antibodies raised against denatured-reduced C3 were inhibited by a lower dose f C3(SD) than of C3(SN). In conclusion the specificity of both types of mouse anti-C3 antisera was mainly for C3(S) antigens.

Specificity of monoclonal antibodies for soluble antigens

The monoclonal antibodies of groups I - III were tested individually in the inhibition ELISA for binding to 25 pmol of C3 expressing C3(D), C3(SD) or C3(SN) antigens in fluid-phase. As shown in figure 2, group I was only inhibited by C3(SN) and not by C3(SD) or C3(D) antigens, which indicated that C3(N) antigens of native C3 caused the inhibition.

Group III was also clear-cut in that C3(D) and C3(SD) affected binding, due to the presence of C3(D) antigens. The limited inhibition caused by native C3 was probably due to a small fraction of molecules denatured during preparation and storage and the dose of antigen needed to obtain an identical degree of inhibition by C3(SN) as by C3(D) was more than 32 times higher.

Group II was more complex since the antibodies were inhibited by all forms of C3. Three of the antibodies were preferentially inhibited by C3(D) antigens while others were more affected by C3(SN) and C3(SD) antigens, suggesting that the specificity of group II antibodies was for both C3(S) and C3(D) antigens.

The specificity of the monoclonals for 2.5 pmol of soluble fluid-phase fragments expressing S and N antigens (C3b, C3bi, serum, aged serum) was also tested (figure 3). The different preparations affected the binding of each group in a similar way. Group I was considerably inhibited by all fragments while group II presented a heterogeneous pattern with a wide range of degrees of inhibition. Group III was only moderately affected by the different fragments with the exception of 4 antibodies that were inhibited to some degree by C3bi.

Specificity of monoclonal anti-C3 antibodies for particle-bound C3

The antibodies of groups I - III were tested individually by inhibition ELISA against 2.5 pmol of particle-bound C3 of $EAC14^{oxy}23b$, $EAC14^{oxy}23bi$ and ZyC3bi (figure 4). Particle-bound C3 effected inhibition (<90 % binding) of all antibodies of group I. The mean level of binding was lowest when $EAC14^{oxy}23bi$ was used. Eight to nine antibodies from group II were affected by particle-bound C3 and the mean values were identical. Eight antibodies of group III bound to bound C3. Of those only one reacted with both particle-bound C3b and

C3bi and the rest specifically with C3bi. Group III antibodies that had been shown to be inhibited by soluble physiological fragments of C3 were preferentially inhibited by the bound form since a 64 - 256 times higher dose of the soluble fragments was needed to effect an identical level of inhibition in the inhibition ELISA.

Four of the antibodies specific for C3bi were considered to have a satisfactory specificity for an immune complex assay.

Specificity of monoclonal anti-C3 antibodies for solid-phase C3 and C3 fragments in ELISA

The antibodies of group I - III were tested by direct-binding ELISA for binding to preadsorbed native C3, SDS-denatured C3, protease-generated C3 fragments and C3 alpha and beta chain (Table I). All of the antibodies of group I bound to native but only one to SDS-denatured C3, 12 bound to C3c and 2 to the C3 alpha or beta chain. In contrast to group I all antibodies of groups II and III bound to both native and denatured C3 and the majority was positive for binding to the C3 alpha chain, but the distribution of binding to the protease-generated fragments was similar to group I antibodies.

TABLE I

Specificity of groups I - III for different forms of C3 on the solid-phase in ELISA

	C3(SN)	C3(SD)	C3c	C3d	C3a	C3(D) alpha	C3(D) beta
I	14	1	12	0	0	1	1
II	14	14	10	2	1	11	0
III	13	13	9	1	0	10	0

Specificity of monoclonal anti-C3 antibodies for reduced C3 fragments in immunoblotting

In immunoblotting (Table II) group I was almost completely unreactive except for one monoclonal that bound vaguely to the beta chain. Group II also bound rather poorly since only 7 out of 14 were found to be positive. With one exception these antibodies bound to the 36 kd fragment of elastase-generated C3c. All antibodies of 13 from group III bound with high affinity to alpha chain fragments. As many as 7 monoclonals bound to the 25 kd fragment and 4 to the 36 kd fragment of elastase-generated C3c and one to elastase-generated C3d(19). One antibody was positive for the 40 kd alpha chain fragment created upon SDS-denaturation at 100 ^{o}C (28) but not for reduced or unreduced C3c or C3d in immuno-blotting, suggesting that it might bind to the C3g fragment (11).

TABLE II

Specificity of groups I - III for reduced fragments in immunoblotting

	C3		C3bi	C3c		C3d
	alpha	beta	70 kd	36 kd	25 kd	
I	0	1?	0	0	0	0
II	6	0	1	6	0	0
III	13	0	ND	4	7	1

Summarized specificity of anti-C3(D) antibodies from groups II and III

Monoclonal antibodies which were preferentially inhibited by C3(D) antigens expressed by denatured and denatured-reduced C3 as shown in figure 2, were defined as anti-C3(D) antibodies. Three monoclonal antibodies with anti-C3(D) specificity from group II and 13 from group III were selected and their properties are summarized in Table III. All of them bound to alpha chain fragments. Those that bound to the 36 kd fragment of C3c were, with one exception, completely unreactive with bound C3. The one that was positive bound to both C3b and C3bi. As many as 7 antibodies bound to the 25 kd fragment and interestingly 6 of these bound specifically to particle-bound C3bi, as was the case with the monoclonal antibody that was presumed to bind to C3g. The antibody that bound to C3d was unreactive with bound C3.

TABLE III

Summarized specificity of anti-C3(D) monoclonal antibodies selected from groups II and III

Inhibition ELISA				Immunoblotting			
Particle-bound		Soluble		C3c		C3d	Group
C3b	C3bi	C3b	C3bi	25 kd	36 kd		
-	-	-	-	-	+	-	
-	-	-	-	-	+	-	II
-	-	-	-	-	+	-	
-	-	-	-	-	+	-	
-	-	-	-	-	+	-	
+	+	-	-	-	+	-	
-	+	-	-	+	-	-	
-	+	-	-	+	-	-	
-	+	-	-	+	-	-	III
-	+	-	-	+	-	-	
-	+	-	-	+	-	-	
-	+	-	-	+	-	-	
-	+	-	-	+	-	-	
-	-	-	-	+	-	-	
-	-	-	-	-	-	+	
-	+	-	-	-[1)]	-	-	

1) Binding to the 40 kd fragment created upon SDS-denaturation at 100 °C.

Immune complex analysis in plasma samples

Several human plasmas were analyzed for the sake of obtaining a preliminary rough idea as to which levels of the immune complexes are still detectable, as to how they are distributed

in a group of patients on one hand and in normal individuals on the other hand, and as to how they may differ inter se when detected on the basis of different neoantigens on C3.

A minor group of patients whose plasma samples had been delivered for analysis of Clq-binding immune complexes were compared with plasma from a minor group of healthy blood donors. The group of patients is a heterogeneous mixture of suspected immunological disorders, so what may be expected are immune complexes of various compositions and in varying amounts. Since a positive correlation with the Clq analysis (15) was neither expected nor detected, all the plasma samples were analysed which had been delivered during a given period of time for Clq analysis. This was done irrespective of whether the Clq analysis was positive or negative.

Three monoclonals were selected, adsorbed to microtiter wells, and employed for separating off immunocomplexed C3 from plasma. One of these monoclonals was directed against a neoantigen that is exposed in bound C3b and C3bi and is located in the C3c alpha fragment of 40 kd molecular weight. A second monoclonal was directed against a neoantigen that is exposed in bound C3bi but is not exposed in bound C3b, and is believed to be directed against the g portion of C3d,g. Both of these belong to the group III monoclonals. The third one of the monoclonals belonged to group II, was directed against the 20 kd fragment of C3c and reacted with bound C3bi.

Statistical treatment e.g. for the purpose of determining the limits for normal values has not been carried out, the material being so small and heterogeneous. In particular the patients do not present any typical normal distributions. A look at the charts of Figs. 5.1 to 5.6 will reveal considerable irregularities in distributions and superimpositions occur

in several places. Also, the range of variation is greater among the patients, which might possibly be interpreted as showing that some of the values are abnormal.

The fact that there is a group of patients lying on a lower level than normal individuals is interesting in itself and indicative of a high sensitivity of the system. Earlier anti-C3-based analyses (1, 30) and also several other immune complex analyses (14, 15) have the bulk of their normal values and many of their patients' values lying on the lowest detectable level. In particular, the lower values in patients than in normal individuals are an unusual phenomenon. If, departing from the one-dimensional view, one proceeds to plotting values from analyses with two different monoclonals into a two-dimensional system of coordinates then one will find that the differences between normal values and patients' values are amplified (Fig. 6). The variation in reactivity with respect to a plurality of epitopes is supported by the theory that the "immune complex" is a broad, summary concept encompassing a sizable amount of heterogeneity (15). The pluridimensional presentation makes it possible to distinguish for instance (a) a high quota of immunocomplexed C3d in plasmas containing low immune complex levels and therefore having a relative accumulation of metabolites deriving from a later stage of the elimination process, from (b) a plasma in which the C3d quota is more normal.

From the analyses of the fourteen C3(D) monoclonals of group III it may be assumed that also the rest of the expanded monoclonals will react preferentially with epitopes on C3(D) in solution, and that they will react to a large extent with neoantigens occurring on physiological forms of particle-bound C3. A significant portion of these neoantigens are probably to be found on C3 associated with immune complexes or models thereof in the form of aggregated human IgG. Moreover, they vary inter se in their specificities for various types of immunocomplexed C3. As shown by preliminary tests they exist in at least two variant forms. For this

reason they can detect different aspects of immunocomplexed C3, and in combination they can improve the resolution and quality of the information. It is not unreasonable, therefore, to assume that the remaining clones of group III may contain antibodies which are directed against other neoantigens highlighting other properties of the immune complexes.

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Figure 1: Serial dilutions of C3(SN)(O), C3(SD)(•) and C3(D) (Δ) were allowed to compete for binding to rabbit polyclonal anti-C3(D)(I) and antisera raised in mice against SDS-denatured (II) and denatured-reduced C3(III) in the inhibition ELISA.

Figure 2: 25 pmol of C3(SN), C3(SD) or C3(D) was allowed to compete for binding to the individual monoclonal antibodies of groups I - III in the inhibition ELISA.

Figure 3: 2.5 pmol of C3b, C3bi, C3 of normal human serum (NHS) and C3 fragments of aged human serum (AHS) was allowed to compete for binding to the individual monoclonal antibodies of groups I - III in the inhibition ELISA. The mean values ± SEM for the different groups in response to each C3 preparation were presented.

Figure 4: 2.5 pmol of particle-bound C3 of $EAC14^{oxy}23b$, $EAC14^{oxy}23bi$ and ZyC3bi was allowed to bind to the individual monoclonal antibodies of groups I - III in the inhibition ELISA.

Figure 5: Histogram showing percent distribution of patients material (a) and normal material (b) when analyses are carried out using the three monoclonal examples mentioned in the text. The antibody in analysis I is directed against a neoantigen exposed on bound C3b and C3bi and located in the C3c portion. In analysis II, the antibody is directed against a neoantigen exposed on bound C3bi, located in C3d,g, and in analysis III the antibody is directed against a neoantigen exposed on bound C3bi located in the C3c portion.

Figure 6: Analysis responses from the same analyses as in Fig. 5 plotted against each other. Normal material is represented by filled-in rectangles, and patients material is represented by empty circles.

Figure 6.1: Analysis responses I and II plotted against each other.

Figure 6.2: Analysis responses II and III plotted against each other.

Claims

1. Antibody preparation directed against an individual neoantigen in the C3b region of human C3, characterized by immunochemically reacting with

   (a) antigenic determinants in the C3b region of the alpha or beta chain of denatured human C3 in which the disulfide bonds have been reduced,

   but not with

   (b) human native C3.

2. Antibody preparation according to claim 1, characterized by immunochemically reacting with at least one region which is comprised within the C3b regions of said chains and which is selected from the group consisting of the C3bi, C3d,g, C3d, C3g and C3c regions.

3. Antibody preparation according to claim 1 or 2, characterized in that the antigenic determinant with which it reacts immunochemically is located in a C3 region corresponding to a physiologically occurring covalently bound C3 fragment on a circulating immune complex.

4. Antibody preparation according to any of claims 1 - 3, characterized in that the antigenic determinant with which it can react immunochemically is not accessible for reaction in soluble (free) physiological C3 or C3 fragments but is accessible for reaction when these fragments are bound to circulating immune complexes.

5. Antibody preparation according to any of claims 1 - 3, characterized by having been produced in that cells potentially capable of producing antibodies - said antibodies reacting immunochemically with both (a) a

neoantigen in human C3 and (b) an antigenic determinant in the C3b region of the alpha or beta chain of denatured human C3 in which the disulfide bonds linking said chains together have been reduced - are caused to secrete the antibodies whereupon the antibodies are purified in a manner known per se and optionally also isolated and/or derivatized in a manner known per se.

6. Use of an antibody preparation according to any of claims 1 - 5 for immunochemically binding C3 fragments bearing the neoantigen against which the antibody preparation is directed.

7. Production of an antibody preparation possessing specificity for a neoantigen in the C3b region of human C3, characterized in that cells potentially capable of producing antibodies - said antibodies reacting immunochemically with both (a) a neoantigen in human C3 and (b) an antigenic determinant in the C3b region of the alpha or beta chain of denatured human C3 in which the disulfide bonds linking said chains together have been reduced - are caused to secrete the antibodies whereupon the antibodies are purified in a manner known per se and optionally also isolated and/or derivatized in a manner known per se.

FIG 1

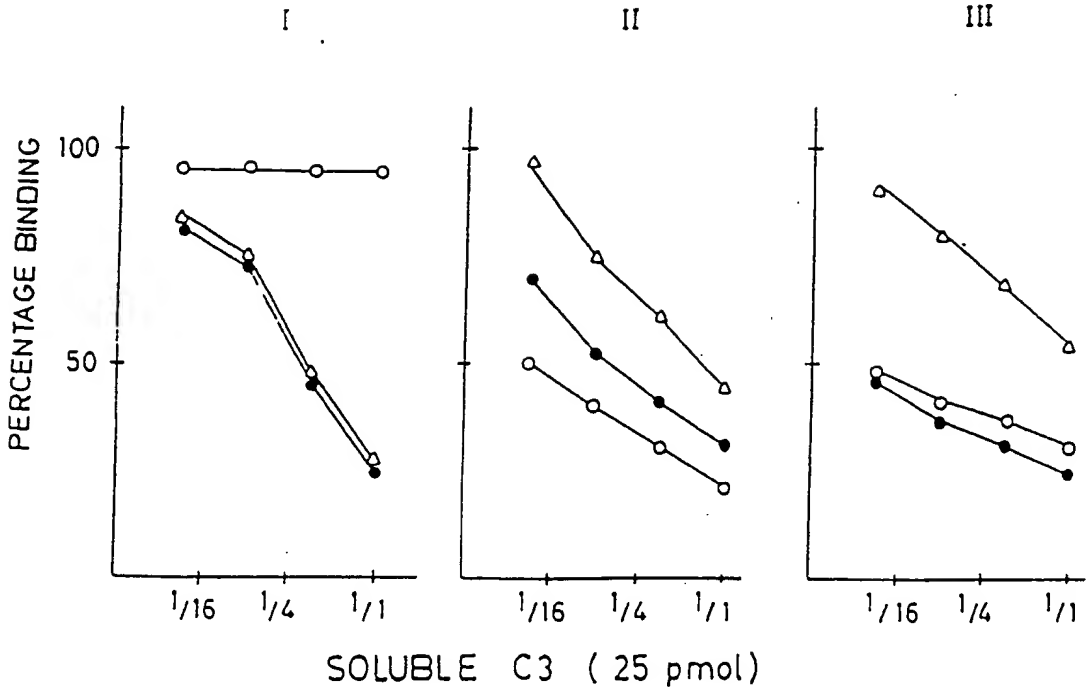

I
II
III
PERCENTAGE BINDING
100
50
1/16
1/4
1/1
SOLUBLE C3 (25 pmol)

FIG 2

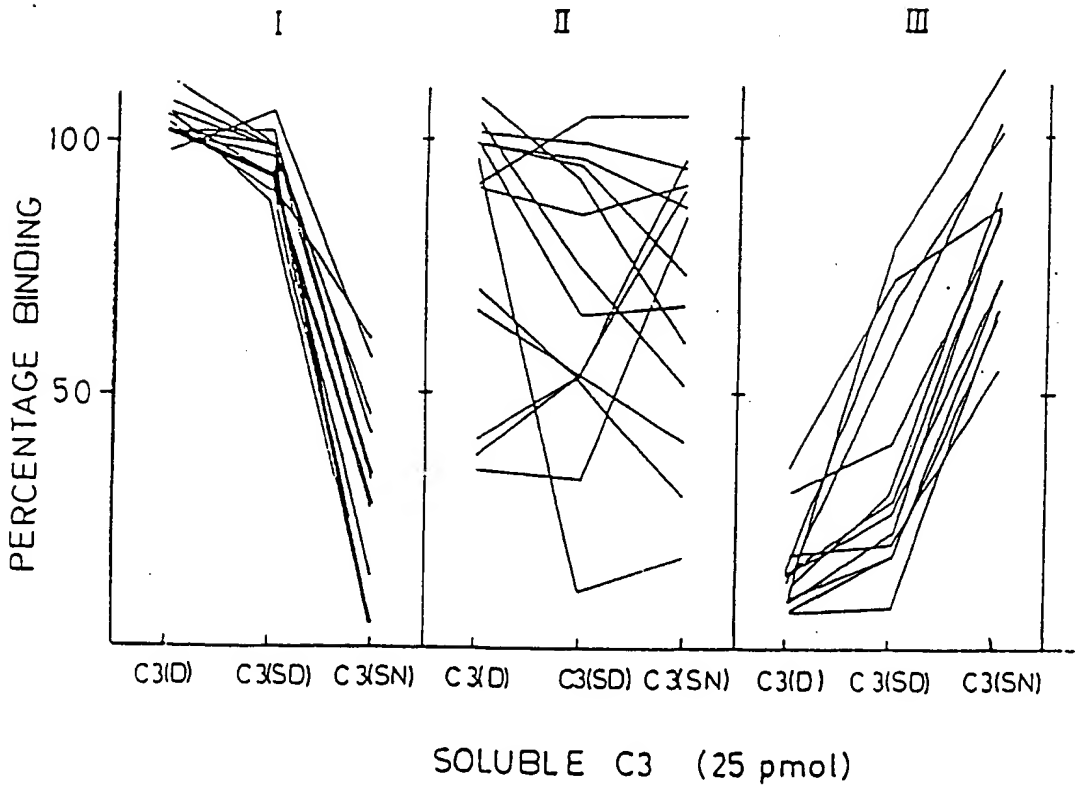

I
II
III
PERCENTAGE BINDING
100
50
C3(D)
C3(SD)
C3(SN)
SOLUBLE C3 (25 pmol)

FIG 3

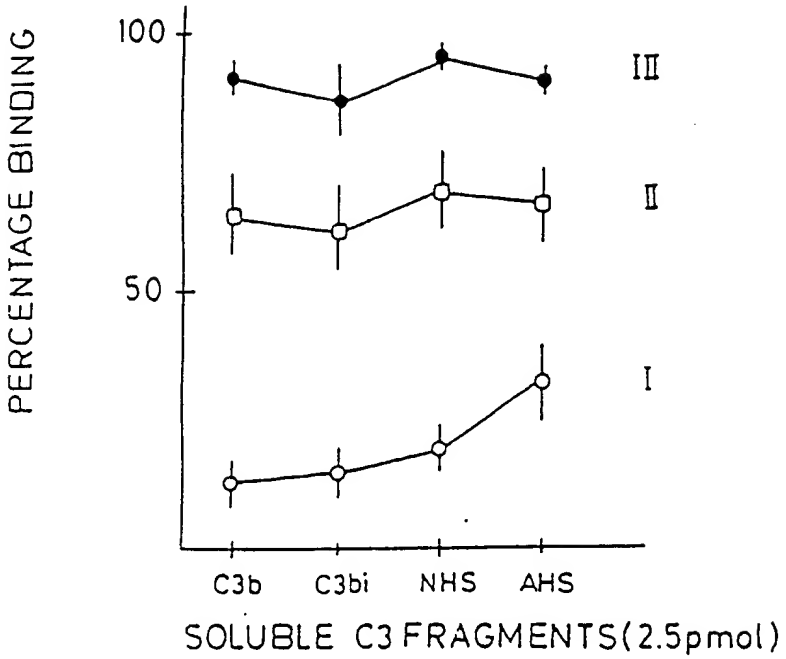

PERCENTAGE BINDING
100
50
III
II
I
C3b
C3bi
NHS
AHS
SOLUBLE C3 FRAGMENTS(2.5pmol)

FIG 4

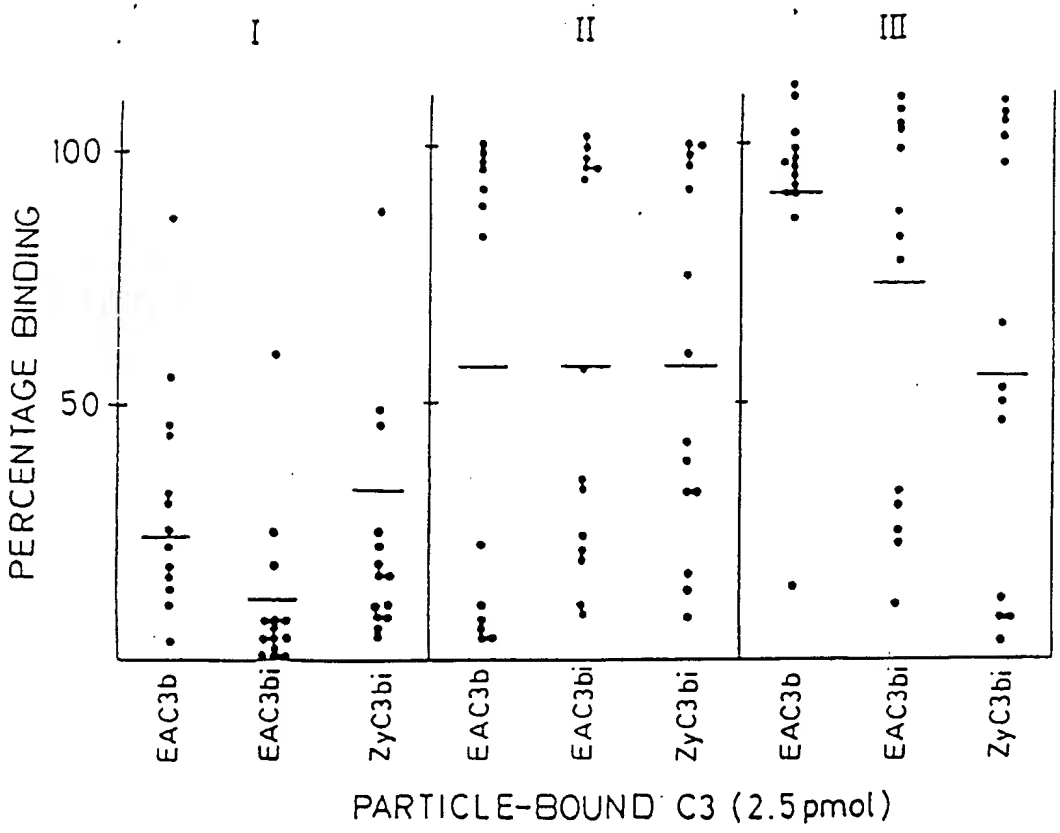

I
II
III
PERCENTAGE BINDING
100
50
EAC3b
EAC3bi
ZyC3bi
EAC3b
EAC3bi
ZyC3bi
EAC3b
EAC3bi
ZyC3bi
PARTICLE-BOUND C3 (2.5pmol)

FIG 5

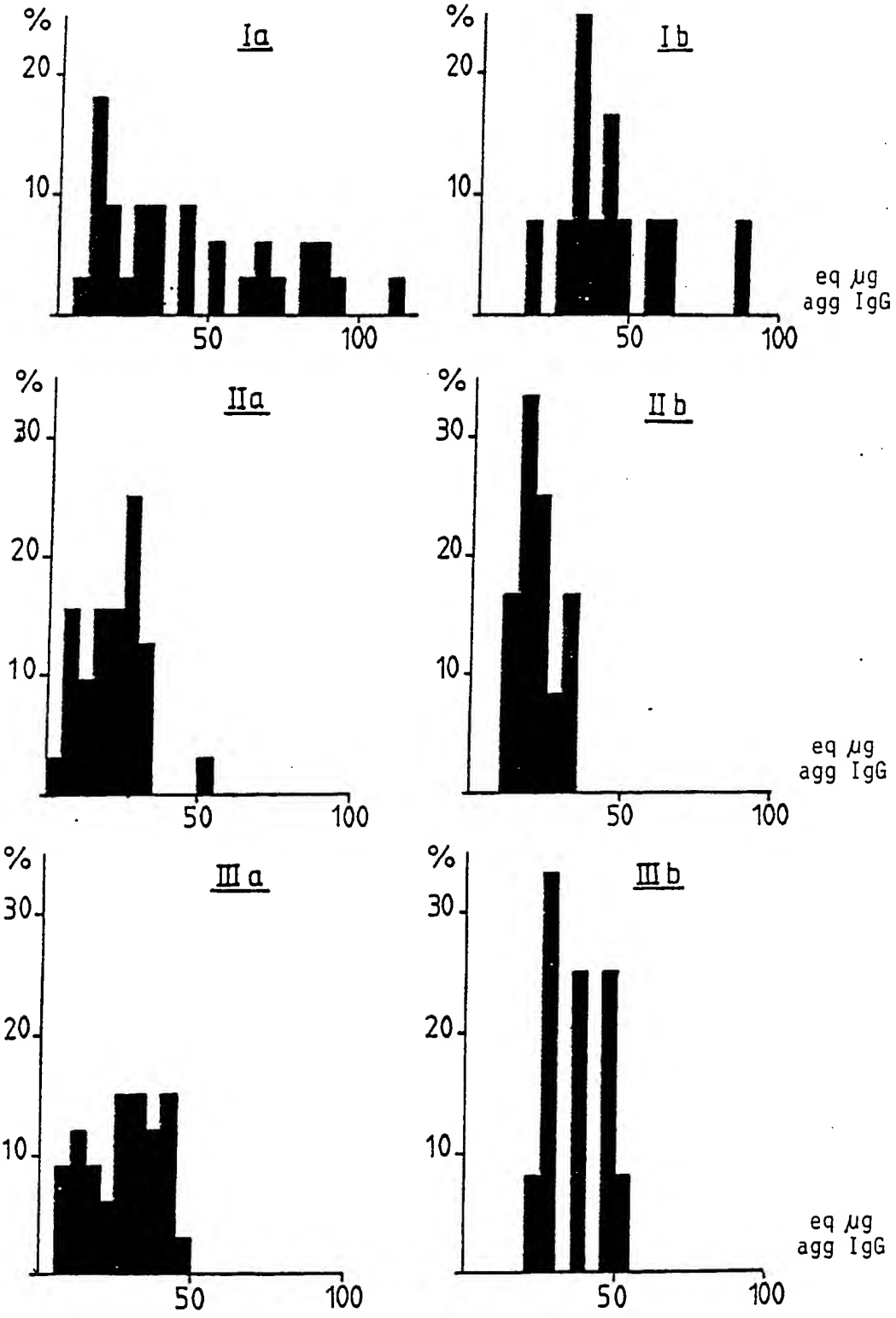


FIG 6.1

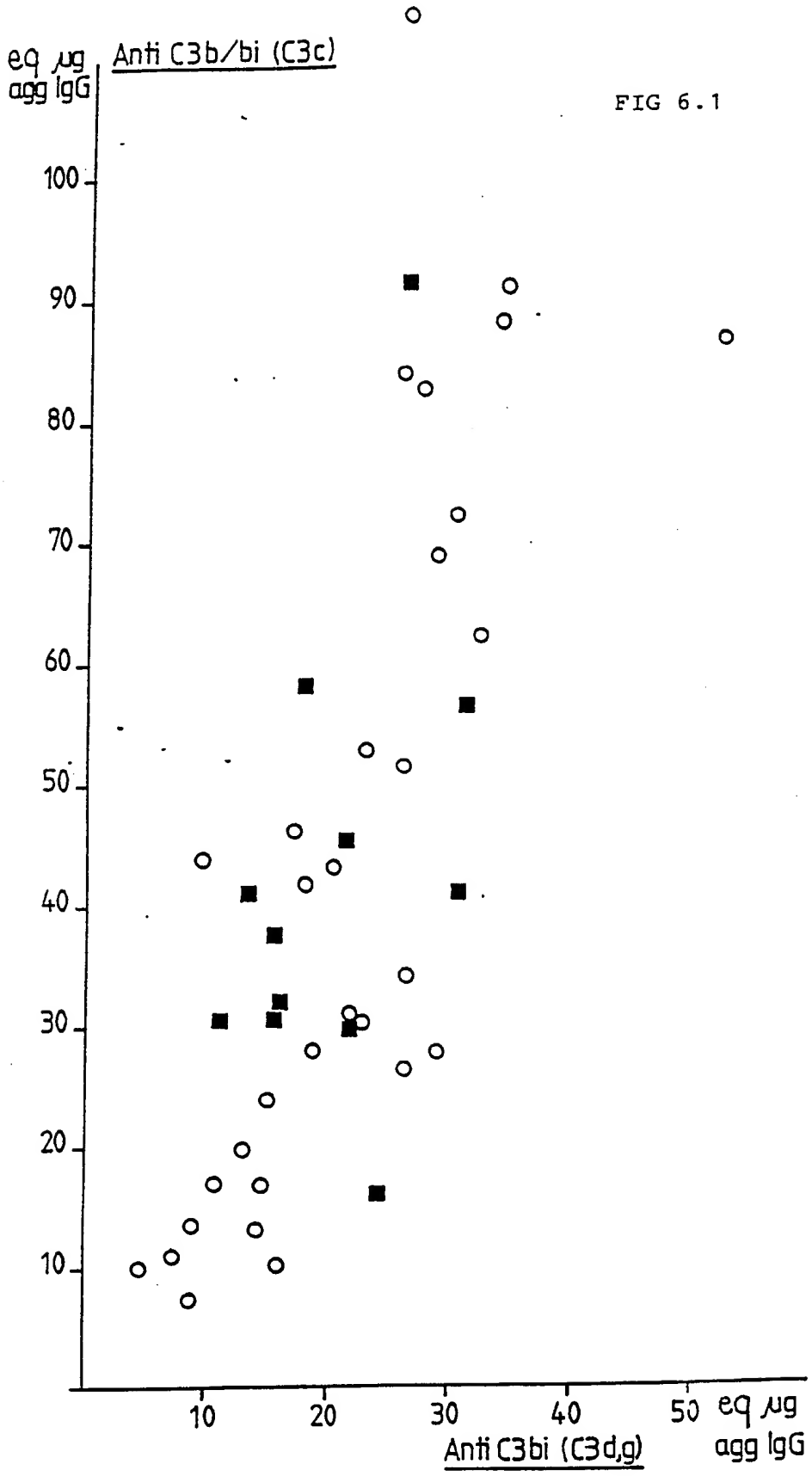
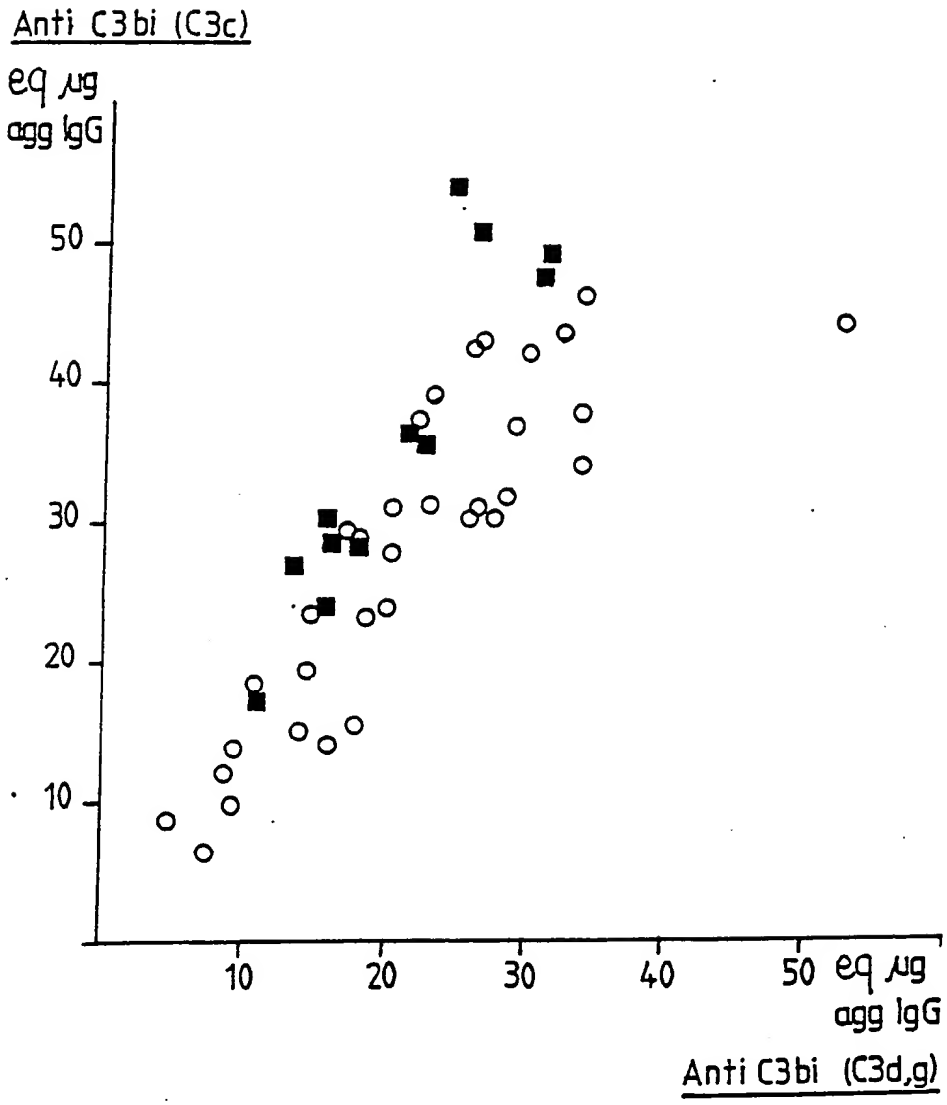

eq µg agg IgG
Anti C3b/bi (C3c)
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
eq µg agg IgG
Anti C3bi (C3d,g)

FIG 6.2


Anti C3bi (C3c)
eq µg
agg IgG
50
40
30
20
10
10
20
30
40
50 eq µg
agg IgG
Anti C3bi (C3d,g)

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE87/00142

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC [4]

G 01 N 33/53, 33/564

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	G 01 N 33/53, /543, /564, /577
US Cl	435: 6,7: 436: 500, 506, 507, 518, 547, 548

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	GB, A, 2 117 514 (EAST ANGLIAN REGIONAL HEALTH 12 October 1983	1-7
A	WO, A1, 81/02469 (SCRIPPS CLINIC & RESEARCH FOUNDATION) 3 September 1981	
A	EP, A2, 128 696 (SCRIPPS CLINIC & RESEARCH FOUNDATION 19 December 1984	
A	Chemical Abstracts, vol 103 (1985), abstract No 69524m, Transfusion (Philadelphia) 1985, 25(3), 267-9	1
A	Chemical Abstracts, vol 103 (1985), abstract No 52 485b, J Exp Med 1985, 161(6), 1414-31	1
Y	J Exp Med, vol 156 (1), page 205-16, published 1982 (Lachmann P J et al) "Breakdown of C3 after Complement Activation Identification of a New Fragment, C3g, Using Monoclonal Antibodies"	1-7

.../...

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
1986-06-17	1987 -06- 23
International Searching Authority	**Signature of Authorized Officer**
Swedish Patent Office	Carl Olof Gustafsson

MJ

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, vol 100 (1984), abstract No 101 357k, J Immunol 1984, 132(2), 809-15	1-7
A	Chemical Abstracts, Vol 100 (1984, abstract No 4 560p, Vox Sang 1983, 44(5), 367-72	1-7
Y	Scand J Immunology, vol 22, page 703-10 published 1985 (Nilsson B, Nilsson U R) "An Assessment of the Extent of Antigenic Analogy beween Physiologically Bound C3 and C3 Denatured by Sodium Dodecyl Sulphate"	1-7
A	The Journal of Immunology, Vol 135, No 3, September 1985, John D Tamerius, "Detection of a Neoantigen on Human C3bi and C3d by Monoclonal Antibody", See particularly p 2018, left col, l 20-21 from below	1-7
Y	Journal of clinical investigation, 76(1985) M Teresa Aguado, "Monoclonal Antibodies against Complement 3 Neoantigens for Detection of Immune Complexes and Complement Activation", see in particular p 1420, right col and tabel 1	1-7
Y	Journal of Immunological Methods, 88 (1986) 33-36, Y Kanayama, "Direct quantitation of Activated C3 in Human Plasma with Monoclonal Anti-iC3b-C3d-Neoantigen", see particularly p 36, lines 8-11	1-7
A	Vox Sang 50: 42-51 (1986), Hugh Chaplin, "Comparisons of Pooled Polyclonal Rabbit Anti-Human C3d with Four Monoclonal Mouse Anti-Human C3ds. I Preparation, Purification and Binding Properties"	1-7
P	Electrophoresis 1986, 7, 379-386, Jørgen Folkersen, "Immunoblotting analysis of the Peptide Chain Structure of the Physiological Breakdown Products of the Third Component of Human Complement"	1-7